



National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 3255

Green Tea (*Camellia sinensis*) Extract

This Standard Reference Material (SRM) is intended primarily for use in validating analytical methods for the determination of catechins and xanthines in extracts of green tea (*Camellia sinensis*) and similar matrices. SRM 3255 can also be used for quality assurance when assigning values to in-house control materials. A unit of SRM 3255 consists of five packets, each containing approximately 1 g of extract.

The development of SRM 3255 was a collaboration among the National Institute of Standards and Technology (NIST), the National Institutes of Health Office of Dietary Supplements (NIH-ODS), and the Food and Drug Administration Center for Drug Evaluation and Research (FDA CDER).

Certified Mass Fraction Values: The certified mass fraction values of selected catechins and xanthines are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in its accuracy in that all known or suspected sources of bias have been investigated or taken into account [1]. Values were derived from the combination of results provided by NIST using two independent methods and by two collaborating laboratories. The certified values in this material are the equally weighted means of the individual sets of results; the associated uncertainties are expanded uncertainties at the 95 % level of confidence [2–4]. Values are reported on a dry-mass basis in mass fraction units [5].

Reference Mass Fraction Values: Reference mass fraction values for epigallocatechin methylgallate, gallic acid, theanine, and theophylline are provided in Table 2. Reference values are noncertified values that are the best estimate of the true values based on available data; however, the values do not meet the NIST criteria for certification [1] and are provided with associated uncertainties that may reflect only measurement reproducibility, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods.

Expiration of Certification: The certification of **SRM 3255** is valid, within the measurement uncertainty specified, until **30 November 2021**, provided the SRM is handled and stored in accordance with the instructions given in this certificate (see “Warning and Instructions for Storage and Use”). The certification is nullified if the SRM is damaged, contaminated, or otherwise modified.

Maintenance of SRM Certification: NIST will monitor this material over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet or register online) will facilitate notification.

Support for the development of SRM 3255 was provided in part by NIH-ODS and FDA CDER. Technical consultation was provided by J.M. Betz (NIH-ODS) and A. NguyenPho (FDA CDER).

Overall direction and coordination of the technical measurements leading to the certification of this SRM were performed by L.C. Sander, K.E. Sharpless, and S.A. Wise of the NIST Chemical Sciences Division.

Acquisition of the material was coordinated by A. NguyenPho of FDA CDER and K.E. Sharpless.

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Analytical measurements at NIST were performed by M. Bedner, J.L. Molloy, K.E. Murphy, B.J. Porter, M.C. Tims, and L.J. Wood of the NIST Chemical Sciences Division. Results were also provided by analysts at two collaborating laboratories: M. Payne at Hershey Foods Corporation (Hershey, PA) and M. Roman at Tampa Bay Analytical Research, Inc. (Largo, FL).

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Office of Reference Materials.

WARNING AND INSTRUCTIONS FOR STORAGE AND USE

Warning: For research use. Not for human consumption.

Storage: The material should be stored at controlled room temperature (20 °C to 25 °C), in an unopened packet, until needed.

Use: Prior to removal of a test portion for analysis, the contents of a packet of material should be mixed thoroughly. For certified values to be valid, test portions of 20 mg to 50 mg for catechin analyses and 20 mg to 100 mg for xanthine analyses should be used. The stability of catechins, xanthines, and theanine in opened packets has not been investigated. Test portions should be analyzed as received and results converted to a dry-mass basis by determining moisture content (described below) on a separate test portion.

PREPARATION AND ANALYSIS⁽¹⁾

Material Acquisition and Preparation: The material for production of SRM 3255 was received as nominally 180 µm (80 mesh) particles and was packaged as received. The extract was transferred to High-Purity Standards (Charleston, SC) where it was blended, aliquotted, and heat-sealed inside nitrogen-flushed 4 mil polyethylene bags, which were then sealed inside nitrogen-flushed aluminized plastic bags along with two packets of silica gel each. Following packaging, SRM 3255 was irradiated (Neutron Products, Inc., Dickerson, MD) to an absorbed dose of 7.9 kGy to 9.5 kGy.

Analytical Approach for Determination of Catechins, Gallic Acid, Xanthines, and Theanine: Value assignment of the concentrations of the catechins, gallic acid, and xanthines in SRM 3255 was based on the combination of measurements from two different liquid chromatography (LC) methods with different detection, i.e., ultraviolet absorbance detection (UV) and mass spectrometry (MS) and by data provided by collaborating laboratories using LC with fluorescence detection (LC/FL) and LC/UV. NIST provided theanine measurements by using LC/MS.

NIST Analyses for Catechins: Catechins were measured by using two LC methods with UV or MS detection. Calibrants were prepared gravimetrically, and a single internal standard solution was used for the calibrants and samples. For Catechin Method 1, below, a series of three calibrants containing varying analyte levels was used. For Catechin Method 2, below, four calibrants were prepared at levels approximating the values expected in the SRMs.

Catechin Method 1. To establish the optimum test portion size for catechin analysis, test portions of varying masses (20 mg to 75 mg) were analyzed. Material from each of six packets was individually combined with 7-(β-hydroxypropyl)theophylline (proxiphylline; the internal standard) and dissolved in methanol and water by shaking for 1 min. Samples were analyzed by using LC/UV with a C₁₈ column and absorbance detection at 210 nm. A typical separation is provided in Figure 1.

Catechin Method 2. Two 20 mg test portions from each of six packets were dissolved in methanol and water by ultrasonic agitation for 5 min. Proxiphylline (internal standard) was added, and samples were analyzed by using LC/UV and LC/MS. A C₁₈ column (different brand than that used for Catechin Method 1) was used with an absorbance detector (detection at 280 nm) and a mass spectrometer (electrospray ionization source; ESI) connected in series. Selected ion monitoring was used for quantitation at m/z 171 for gallic acid (GA), m/z 239 for proxiphylline (internal standard; IS), m/z 291 for catechin (C) and epicatechin (EC), m/z 307 for gallocatechin (GC) and epigallocatechin (EGC), m/z 443 for epicatechin gallate (ECG), and m/z 459 for gallocatechin gallate (GCG) and epigallocatechin gallate (EGCG). A typical separation is provided in Figure 1.

⁽¹⁾Certain commercial instruments, materials, or processes are identified in this certificate to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the instruments, materials, or processes identified are necessarily the best available for the purpose.

NIST Analyses for Xanthines: Xanthines were measured by using LC/UV and LC/MS. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the xanthines in the SRM. A single internal standard solution was used for the calibrants and samples.

Xanthine Method 1. Caffeine and theobromine were measured simultaneously with the catechins measured using Catechin Method 1, above.

Xanthine Method 2. Caffeine and theophylline were measured in two 45 mg test portions from each of six packets. Test portions were individually combined with an internal standard solution containing trimethyl- $^{13}\text{C}_3$ -caffeine and $^{13}\text{C}^{15}\text{N}_2$ -theophylline, methanol, and water. Materials were extracted using ultrasonic agitation for 2 h. Samples were syringe-filtered prior to LC/MS analysis. A C_{18} column and ESI in positive polarity were used, and ions at m/z 198 for labeled caffeine, m/z 195 for caffeine, m/z 184 for labeled theophylline, and m/z 181 for theophylline were monitored.

Xanthine Method 3. Theobromine (and theanine) were measured in two 100 mg test portions from each of six packets. Test portions were individually combined with an internal standard solution containing $^2\text{H}_6$ -theobromine and $^2\text{H}_5$ -L-theanine, methanol, and phosphate buffer in water. Materials were extracted using ultrasonic agitation for 2 h. Samples were syringe-filtered prior to LC/MS analysis. A C_{18} column and ESI in positive polarity were used, and ions at m/z 187 for labeled theobromine, m/z 181 for theobromine, m/z 180 for labeled theanine, and m/z 175 for theanine were monitored.

NIST Analyses for Theanine: Theanine was measured simultaneously with theobromine (Xanthine Method 3) using LC/MS [6]. Calibrants were prepared gravimetrically, at levels intended to approximate the levels of the theanine in the SRM. A single internal standard solution was used for the calibrants and samples.

Collaborating Laboratories' Analyses: Hershey Foods analyzed 250 mg test portions from each of six packets for catechin, epicatechin, caffeine, and theobromine using sonication, and analyzed extracts by using LC/FL (catechins) or LC/UV at 280 nm (xanthines). Tampa Bay Analytical Research analyzed 150 mg test portions in triplicate from each of five packets for catechins and caffeine using sonication. Extracts were analyzed by using LC-UV.

NIST Analyses for Toxic Elements: Two 0.5 g test portions from a single packet of SRM 3255 were screened for arsenic, cadmium, and lead using inductively coupled plasma with mass spectrometric detection (ICP-MS). Three 0.05 g test portions from a single packet were screened for mercury using a mercury analyzer. The material contains an arsenic mass fraction of approximately 160 ng/g, a cadmium mass fraction of approximately 6 ng/g, a lead mass fraction of approximately 80 ng/g, and a mercury mass fraction of approximately 3 ng/g.

Determination of Moisture: Moisture content of SRM 3255 was determined at NIST (see “see “Warning and Instructions for Storage and Use”) by (1) freeze-drying to constant mass over 7 days; (2) drying over magnesium perchlorate in a desiccator at room temperature for 28 days; and (3) drying for 2 h in a forced-air oven at 80 °C. Unweighted results obtained using all three techniques were averaged to determine a conversion factor of (0.9687 ± 0.0055) gram dry mass per gram as-received mass, which was used to convert data from an as-received to a dry-mass basis; the uncertainty shown on this value is an expanded uncertainty. An uncertainty component for the conversion factor (0.28 %) obtained from the moisture measurements is incorporated in the uncertainties of the certified and reference values, reported on a dry-mass basis, that are provided in this certificate.

Homogeneity Assessment: The homogeneity of catechins, xanthines, and theanine was assessed at NIST by using the LC/UV and LC/MS methods described above. An analysis of variance did not show inhomogeneity for the test portions analyzed; see “Warning and Instructions for Storage and Use.”

Value Assignment: The equally weighted mean of results provided by LC-UV, LC/MS, and the individual means of collaborating laboratories' data, where available, were used to calculate assigned values. In cases where data were provided using two detectors in series, the average was treated as a single method mean when it was combined with other data.

Table 1. Certified Mass Fraction Values for Selected Catechins and Xanthines in SRM 3255^(a)

	Mass Fraction (mg/g, dry-mass basis)	<i>k</i>
(+)-catechin	9.17 ± 0.93	2.78
(-)-epicatechin	47.3 ± 6.7	2.92
(-)-epicatechin gallate	100.3 ± 7.8	2.00
(-)-epigallocatechin	81.8 ± 6.5	2.00
(-)-epigallocatechin gallate	422.0 ± 19.0	2.00
(-)-gallocatechin	22.0 ± 1.7	2.00
(-)-gallocatechin gallate	39.0 ± 2.0	2.00
caffeine	36.9 ± 2.7	2.58
theobromine	0.867 ± 0.076	2.00

^(a) Each certified value, expressed as a mass fraction, is an equally weighted mean of results provided by using LC/UV, LC/MS, and the individual means of collaborating laboratories' data where available. In cases where data were provided using UV and MS detectors in series, the average was treated as a single method mean when it was combined with other data. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, as well as a component related to moisture correction, and expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the ISO/JCGM Guide and its Supplement 1 [2–4]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. The measurands are the mass fractions of the selected catechins and xanthines listed in Table 1. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

Table 2. Reference Mass Fraction Values for Epigallocatechin Methylgallate, Gallic Acid, Theanine, and Theophylline in SRM 3255

	Mass Fraction (mg/g, dry-mass basis)	<i>k</i>
(-)-epigallocatechin methylgallate ^(a)	6.87 ± 0.44	2.00
gallic acid ^(b)	3.231 ± 0.086	2.00
L-theanine ^(c)	0.340 ± 0.008	2.00
theophylline ^(c)	0.087 ± 0.002	2.00

^(a) This reference value, expressed as a mass fraction, was obtained by using only LC/UV. The uncertainty provided is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO Guide [2]. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis, as well as a component related to moisture correction. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for this analyte. The measurand is the mass fraction of (-)-epigallocatechin methylgallate as determined by the method indicated. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

^(b) This reference value, expressed as a mass fraction, is an equally weighted mean of results provided by using LC/UV and LC/MS. The uncertainty provided is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence; it incorporates Type B uncertainty components related to the analyses, as well as a component related to moisture correction, and expresses both the observed difference between the results from the methods and their respective uncertainties, consistent with the ISO/JCGM Guide and its Supplement 1 [2–4]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for this analyte. The measurand is the mass fraction of gallic acid as determined by the methods indicated. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

^(c) This reference value, expressed as a mass fraction, was obtained by using only LC/MS. The uncertainty provided is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence, consistent with the ISO/JCGM Guide [2]. The uncertainty incorporates within-method uncertainty and Type B uncertainty components related to the analysis, as well as a component related to moisture correction. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for each analyte. The measurands are the mass fractions of L-theanine, and theophylline as determined by the method indicated. Metrological traceability is to the SI derived unit for mass fraction (expressed as milligrams per gram).

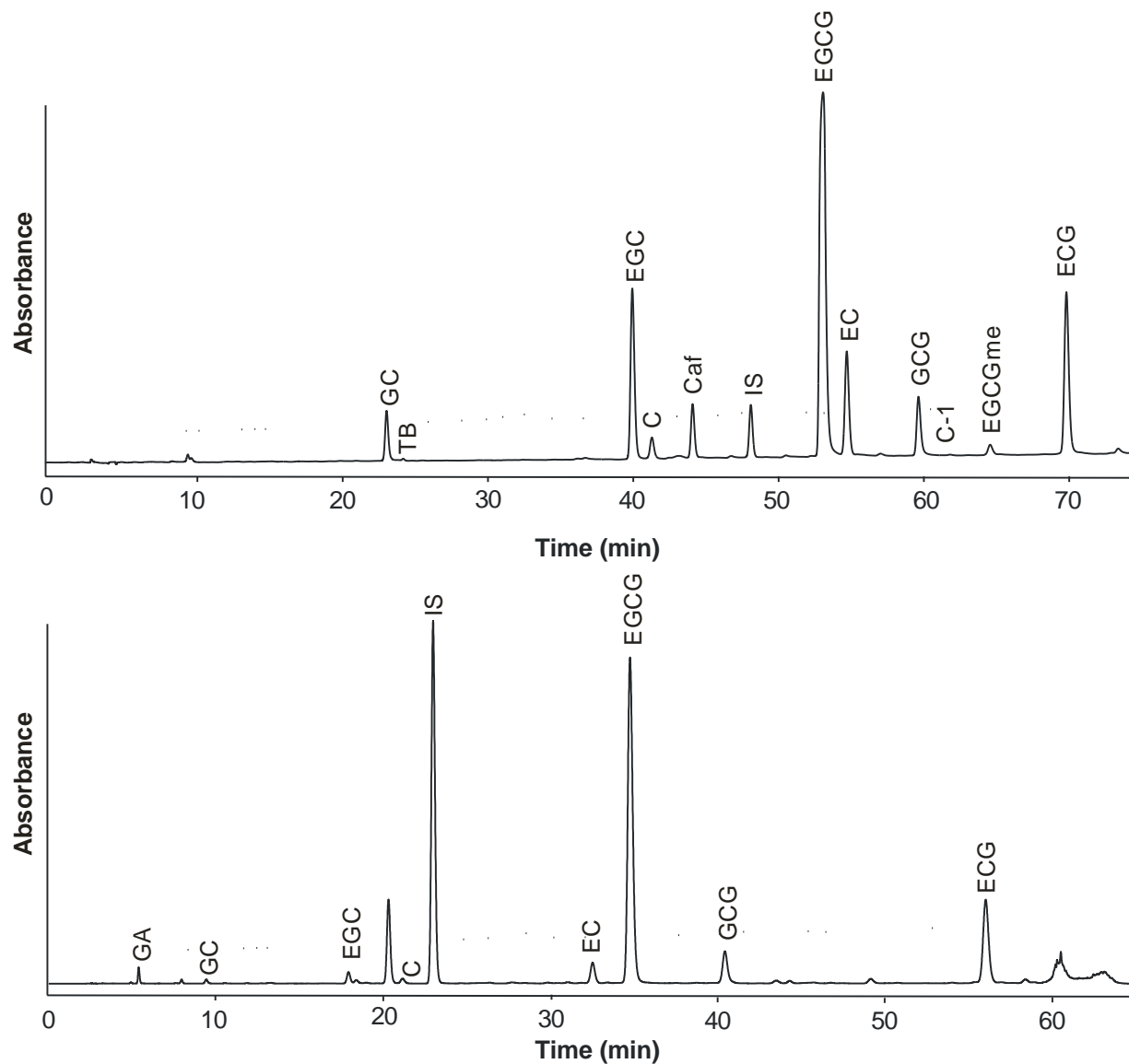


Figure 1. Chromatograms showing separation of catechins, gallic acid, and caffeine using Catechin Method 1 (top) and Catechin Method 2 (bottom). For Catechin Method 1, an Ace C₁₈ ultra inert column (250 mm × 4.6 mm, 5 µm particle size; MAC-MOD Analytical, Chadds Ford, PA) was held at 23 °C. The separation was performed using a gradient consisting of water, acetonitrile, and methanol, each containing acetic acid. The solvent composition reached full elution strength at 75 min. Absorbance detection was at 210 nm. For Catechin Method 2, a Zorbax Eclipse XDB-C18 column (250 mm × 4.6 mm, 5 µm particle size; Agilent Technologies, Palo Alto, CA) was used. The separation was performed using a gradient of water and acetonitrile, both containing 0.1 % formic acid (volume fraction). Absorbance detection was at 280 nm; data were also generated using MS with ESI in positive polarity in series with the absorbance detector (chromatograms not shown). Abbreviations: procyanidin trimer C1 (C-1), caffeine (Caf), catechin (C), epicatechin (EC), epicatechin gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), epigallocatechin methylgallate (EGCGme), gallic acid (GA), galocatechin (GC), galocatechin gallate (GCG), proxyphylline (internal standard; IS), and theobromine (TB).

REFERENCES

- [1] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136; U.S. Government Printing Office: Washington, DC (2000); available at <http://www.nist.gov/srm/upload/SP260-136.PDF> (accessed Dec 2015).
- [2] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at http://www.bipm.org/utls/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Dec 2015); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at <http://www.nist.gov/pml/pubs/tn1297/index.cfm> (accessed Dec 2015).
- [3] JCGM 101:2008; *Evaluation of Measurement Data – Supplement 1 to the Guide to Expression of Uncertainty in Measurement - Propagation of Distributions Using a Monte Carlo Method*; JCGM (2008); available at http://www.bipm.org/utls/common/documents/jcgm/JCGM_101_2008_E.pdf (accessed Dec 2015).
- [4] Efron, B.; Tibshirani, R.J.; *An Introduction to the Bootstrap*; Chapman & Hall, London, UK (1993).
- [5] Thompson, A.; Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at <http://physics.nist.gov/cuu/pdf/sp811.pdf> (accessed Dec 2015).
- [6] Bedner, M.; Sander, L.C.; Sharpless, K.E.; *An LC-ESI/MS Method for Determining Theanine in Green Tea Dietary Supplements*; *Anal. Bioanal. Chem.*, Vol. 397, pp. 1773–1777 (2010).

Certificate Revision History: 30 December 2015 (Editorial changes); 04 September 2015 (Change of expiration date; editorial changes); 15 November 2010 (Original certificate date)

Users of this SRM should ensure that the Certificate of Analysis in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 948-3730; e-mail srminfo@nist.gov; or via the Internet at <http://www.nist.gov/srm>.